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ANALYSIS OF BILE SALT RESISTANCE IN LACTOBACILLUS ISOLATED FROM ICE CREAM

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ABSTRACT

Probiotics are beneficial living microorganisms that can be present in various foods. One is ice cream, a dairy product that can be a food carrier for probiotic bacteria, with Lactobacillus being one of the most prevalent species. Ice cream is a functional and nutritious food that contains valuable components such as dairy, vitamins, and minerals, making the presence of Lactobacillus in ice cream particularly advantageous. The main objective of the research is to offer consumers food that is not only rich in nutritional value but also provides additional probiotic advantages by identifying the Lactobacillus presence and evaluating its resistance to bile salts. In this specific investigation, five ice cream samples from local sources were cultured on a medium formulated specifically for Lactobacillus growth. Following biochemical tests to confirm the existence of Lactobacillus, the samples displayed characteristics such as being Gram-positive, catalase-negative, acid-fast-negative, and endospore-negative. The confirmed Lactobacillus colonies were then subcultured in nutrient broth to analyse bile resistance. The resistance of Lactobacillus to three different concentrations of bile salts (0.1%, 0.2%, and 0.3%) was also evaluated using spectrophotometry at 600 nm. Under control conditions, all samples demonstrated moderate to high growth. Statistical analysis revealed that the differences in bacterial growth across various bile salt concentrations were not statistically significant (p-value > 0.05). Taken together, findings of this study indicates Lactobacillus bacteria isolated form the ice cream samples are resistance to bile salts, underscoring the advantage of utilizing Lactobacillus bacteria to deliver probiotic benefits to consumers.

Keywords: Ice Cream, Probiotics, Lactobacillus, Bile salt resistance

INTRODUCTION

Probiotics is a modern term meaning "for life" and it refers to bacterial communities that have favourable impacts on both human and animal wellness. Such bacteria-containing foods are reasoned to be functional foods. According to Granato et al. (2010), these foods are considered "foods claimed to have a positive effect on health". Functional foods must include at least 106-107 cfug−1 probiotic bacteria at the time of purchase. After initial difficulties, research into probiotics has evolved significantly over the last 20 years, with considerable breakthroughs in the selection and characterisation of specific probiotic cultures, resulting in significant health advantages when consumed (Rout et al., 2018). Early research primarily focused on identifying and isolating probiotic strains that could survive the acidic environment of the stomach. Over time, scientists have developed advanced techniques to ensure these bacteria not only survive but also thrive within the gastrointestinal tract. Recent studies have expanded to investigate how probiotics can influence mental health, immune response, and even metabolic processes (Dempsey and Corr,

2022). Lactic acid bacteria, such as Lactobacilli species, are the most often utilised probiotic agents among the many more microorganisms considered probiotics (Gauri and Morya, 2017).

Probiotics play a key role in the prevention and treatment of various clinical disorders, enhancing the intestinal milieu, initiating immunological modulation, reducing physiological stress, decreasing pathogen development, and increasing intestinal epithelial barrier integrity (Ma et al., 2022; Greeff, 2016). Probiotics have been reported to exert therapeutic effects, primarily through the normalization of intestinal microbiota composition and immunomodulation. This helps in the suppression of both endogenous and exogenous pathogens, thus reducing the occurrences of antibiotic-associated and travelers' diarrhea. By maintaining a balanced immune response, probiotics contribute to the control of irritable bowel syndrome (IBS) and inflammatory bowel diseases (IBD). They also play a role in alleviating food allergy symptoms in infants and strengthening innate immunity. Metabolically, probiotics aid in lowering serum cholesterol through bile salt deconjugation and secretion, improving lactose tolerance via lactose hydrolysis, and reducing risk factors for colon cancer by supplying short-chain fatty acids (SCFA) and vitamins like folate to the colonic epithelium. Additionally, they help in lowering toxic and mutagenic reactions in the gut (Gauri and Morya, 2017; Kumar et al., 2016; Lye et al., 2016).

Lactobacillus is a facultatively anaerobic, catalase-negative, Gram-positive, non-spore-forming bacillus that thrives in microaerobic environments. Lactobacilli show as white, viscous colonies on a variety of different media, including MRS (Mann, Rogosa, and Sharp) agar although their colony morphology on blood agar generally varies from tiny to medium grey colonies with alpha hemolysis. Their gram-stain morphology is diverse, ranging from short, bulky rods to long, thin rods, chains, and palisades. Lactobacillus species are often identified at the molecular level (16S rRNA gene), as morphological identification is often erroneous. Lactobacilli have long been incorporated into the production of dairy products as they exhibit considerable tolerance for extremely low pH levels, which aids their passage through the stomach. Moreover, trace amounts of Lactobacilli create antioxidants (Goldstein, Tyrrell and Citron, 2015).

Resistance to stomach acidity, bile acid resistance, adhesion to mucus and/or human epithelial cells and cell lines, antibacterial activity against potentially pathogenic bacteria or fungus, decreased adherence of surface pathogens, and bile salt hydrolase activity are all crucial features for Lactobacillus to be recognized as a probiotic (Fijan, 2014). Additionally, probiotics tend to increase antioxidant activity and are used as a supplement in the treatment of cancer, allergies, lactose intolerance, vaginosis, and Helicobacter pylori infection (Yadav, Puniya and Shukla, 2016). The effectiveness of probiotic bacteria in food products depends on their ability to survive the harsh conditions of the gastrointestinal tract, particularly bile and acid exposure. A dairy product with lots of potential to serve as probiotic bacteria's food carrier is ice cream. The incorporation is particularly advantageous since, in addition to being a functional and healthy meal, ice cream already contains beneficial ingredients including dairy raw materials, vitamins, and minerals and is consumed by the public. Ice cream, unlike fermented dairy products, is a great matrix for delivering probiotic organisms to the human body. With the pH being closer to neutral than fermented dairy, a low pH helps alter the survival and metabolic activity of probiotic bacteria (Akin, Akin, and Kirmaci, 2007).

Moreover, ice cream supports significantly higher viability of probiotic strains during manufacture and especially during storage than fermented milks do. (Mohammadi et al., 2011; Ranadheera et al., 2017; Kozłowicz et al., 2019). According to Hekmat and McMahon (1992), probiotic ice cream could be manufactured by fermenting an ice cream mix with L. acidophilus. The significant amount of alive organisms detected even after 17 weeks of frozen storage implies that ice cream might be a reliable source of probiotic bacteria for customers. The liver produces bile, which helps dissolve various solid components within the body, including bile salts, bilirubin, phospholipids, cholesterol, amino acids, vitamins, steroids, and enzymes. It is essential for lipid metabolism because it can transport exogenous lipophilic substances and environmental toxins and eliminate excess cholesterol. Bile salt is the primary organic compound found in bile, where it aids in the emulsification of fat to enhance the absorption of cholesterol and fat-soluble vitamins. Furthermore, bile salts also hinder the growth of harmful bacteria in the gut and stifle the creation of endotoxins (Ren et al., 2021). The bile salt pool is carefully controlled under normal conditions but can become inadequate under cholestatic conditions. Increased levels of potentially harmful hydrophobic bile salts in serum and tissues during cholestasis can lead to mitochondrial damage, apoptosis, or necrosis in vulnerable cell types (Maillette de Buy Wenniger and Beuers, 2010).

Overall, the bile response is a complex phenomenon, with the most prevalent bile-specific mechanisms promoting resistance in both genera appearing to be active efflux of bile acids/salts, hydrolysis of bile salts, and changes in cell membrane and wall structure/composition. In addition to bile exposure, general stress responses, protection from oxidative damage, and global glycolytic rearrangement can be used to offset part of the cellular damage produced by these chemicals (Ruiz, Margolles and Sánchez, 2013).

The main element that affects toxicity is bile salt concentration. Elevated concentrations of bile salts induce fast cell membrane disintegration, acidification of the cytoplasm, DNA/protein damage, dissociation of integral proteins, destruction of glucose absorption, and eventually cell death. Bile acids undergo conjugation and deconjugation processes facilitated by bacterial bile salt hydrolase (BSH) enzymes. This leads to the production of free bile acids, which can cause intracellular acidification in bacteria. Bacteria maintain homeostasis by regulating the lipid profile, autoaggregation, and extracellular polysaccharide (EPS) production. They also manage up and down regulation in the biosynthesis of nitrogenous bases, lipids, and amino acids, which are crucial for DNA repair proteins and can influence DNA damage. The catabolism of glucose via the bifid-shunt pathway generates energy. ATP-ase enzymes play a role in bile efflux, using energy from ATP hydrolysis. These processes collectively help maintain bacterial stability and functionality within the intestinal environment (Bustos et al., 2018). Bacteria commonly combat bile toxicity by actively effluxing bile acids and salts that have collected in the cytoplasm and are released through efflux pumps (Piddock, 2006). Bile tolerance in Lactobacillus strains has been characterised as being mediated by several multidrug transporters (MDRs) from the ATP-binding cassette or major promoter superfamily (Pfeiler and Klaenhammer, 2009).

A de novo protein synthesis process and an inducible pre-existing system co-exist to defend against acid stress, according to studies with bile-resistant L. acidophilus variations. Bile salt conjugation has been proposed as one of several defences adopted by bacteria to counteract the adverse effects of bile. This defence is

provided by the action of BSH. Glycine and taurine are separated from bile salts in processes that are facilitated by BSH, and the resulting unconjugated acids are then digested further by different gut bacteria (Jones et al., 2008; Kusada, Morinaga and Tamaki, 2021). Bile and acid stress frequently trigger a variety of resistance mechanisms that modify the Lactobacilli cell surface structures. Cell integrity is maintained in these high-stress settings in part by the cell wall and membrane. Studies have demonstrated that bile salts and cholesterol cause modifications to the lipid cell membrane of L. reuteri (Sengupta et al., 2013). Probiotics in the human gastrointestinal tract allow the body to maintain a healthy microbiome that influences the human immune response. This study aimed to determine the significance of bile salt tolerance for the colonization and survival of the identified Lactobacillus isolated from 5 different commercially available ice cream samples, enabling manufacturers to create high-quality products that can withstand elevated bile salts.

METHODOLOGY

Sample Preparation

Five samples of commercially produced ice cream were obtained and labelled A, B, C, D & E respectively.

Culturing of Sample

Using the quadrant streak method, the five samples were inoculated onto five respectively labelled petri plates with prepared MRS agar. 48 hours were subsequently spent incubating the culture plates at 37°C.

Initial identification of Lactobacillus

Biochemical tests namely Catalase test and staining tests; Gram staining, Acid Fast staining, and Endospore staining were performed to identify the cultured bacteria.

Bile Salt Resistance Assay

In labelled 15mL falcon tubes, bile salt required for concentrations of 0.1%, 0.2%, and 0.3% were dissolved in 5mL of grown subcultures of the five samples. The inoculated cultures were then incubated at 37°C for 24 hours. To assess resistance, the bacterial growth after incubation was evaluated by calculating the mean absorbance at each concentration using a spectrophotometer calibrated at 600nm wavelength. The control cultures were likewise kept and incubated without the addition of any bile salts (Nawaz, Jagadeesh and Krishnaraj, 2017).

Statistical Analysis

Bile salt resistance of Lactobacillus isolated from ice cream products was statistically analysed using one-way ANOVA via SPSS software. P-values less than 0.05 were considered as statistically significant.

RESULTS

Colony morphology

Colony formation of samples cultured on MRS agar observed after 48-hour incubation are shown below.

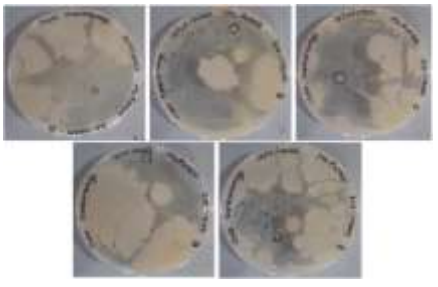

Figure 1: Colonies obtained from streaking of Ice cream samples on MRS Agar

On agar, creamy white colonies with an entire margin with flat raised and convex elevation were visible.

Gram staining

Gram Staining was carried out on the selected colony from each sample to obtain the below results.

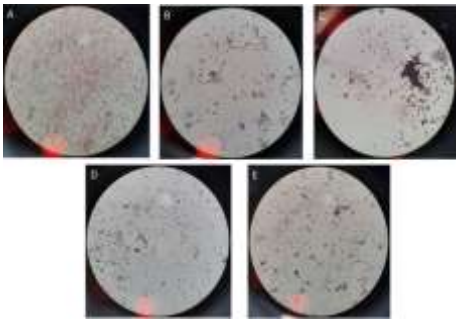

Figure 2: Grams Staining results of Sample A-E observed under oil immersion (x100)

All five samples exhibited purple rod-shaped bacteria, indicating that the bacterium was gram positive.

Catalase test

Catalase test was carried out on the selected colony of each sample to obtain below result.

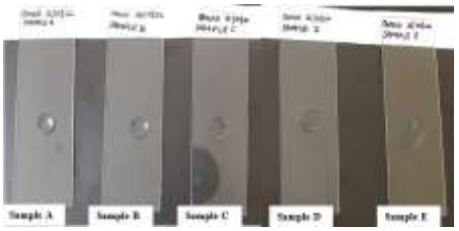

Figure 3: Catalase test for Ice cream samples A-E

No evolution of bubbles of oxygen observed upon addition of H2O2 in all 5 samples indicating that the bacterium present was catalase negative.

Acid Fast Staining

Acid fast staining results of the samples are shown below.

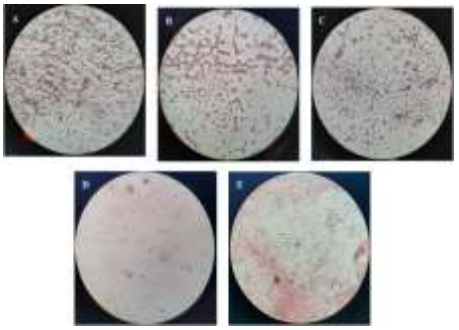

Figure 4: Acid-Fast Staining results of Sample A-E observed under oil immersion (x100)

Blue rod-shaped bacteria were observed in all five samples once stained indicating that the bacterium isolated was acid-fast negative.

Endospore Staining

Results of endospore staining of samples are shown below.

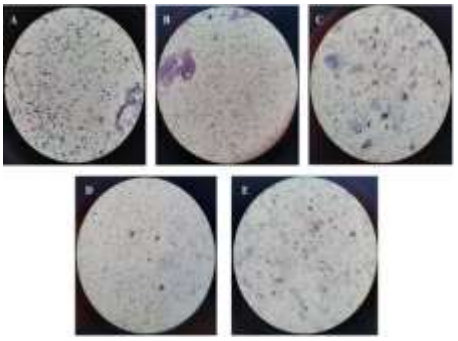

Figure 5: Endospore Staining results of Sample A-E observed under oil immersion (x100).

Pink rod-shaped bacteria were observed in all five samples indicating endospore negative, vegetative bacteria.

Bile Salt Resistance

The assay was conducted to quantify the bile salt resistance capacity of bacteria,

with the use of three bile salt concentrations; 0.1%, 0.2% and 0.3%.

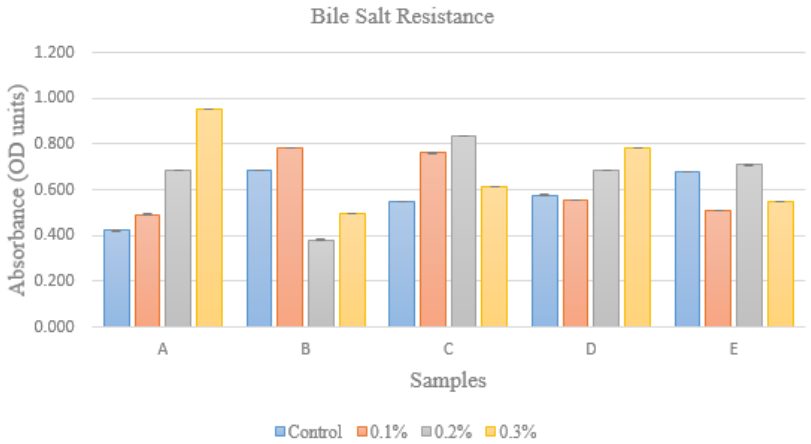

Figure 6: Bile salt resistance of Samples A-E at the three bile salt concentrations 0.1%, 0.2% and 0.3% along with the control.

Sample A and D exhibited significant resistance to bile salts at a concentration of 0.3%, while Sample B displayed notable resistance at 0.1% bile salt concentration. On the other hand, Samples C and E demonstrated significant resistance at a bile salt concentration of 0.2%.

ANOVA Analysis


ANOVA

Absorbance

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.027	4	.014	.576	.565
Within Groups	.996	42	.024		
Total	1.024	44			

Figure 7: ANOVA analysis of bile salt resistance

The obtained p value was greater than 0.05 which indicates there are no significant difference in the absorbances of samples A-E between bile salt concentrations of 0.1%, 0.2% and 0.3%.

DISCUSSION

The research was carried out to identify the presence of Lactobacillus in ice cream samples found locally, and to investigate its capacity for bile salt resistance. According to a study conducted by Haghshenas et al. (2015), on the MRS agar growth medium, the isolated single colonies were hemispherical and white or yellow in hue while Goldstein, Tyrrell and Citron (2015), states that Lactobacilli can also be grown on MRS agar medium, where they appear as white, mucoid colonies. Considering this information, the colonies formed as shown in Figure 1 may be Lactobacillus, as equivalent morphology was detected in the colonies grown on MRS agar in this investigation. For the prevention of fungal growth, Amphotericin B was mixed with the MRS agar, as Amphotericin B and echinocandins are the only two groups of medications that have been demonstrated to be effective in vitro against microbial biofilms (Zangl et al., 2020). Gram staining, catalase test, acid fast staining, and endospore staining were performed to identify Lactobacillus in ice cream samples which showed the presence of Lactobacillus in all the ice cream samples.

The isolated Lactobacillus colony was then sub cultured to promote growth and was used for analysis of characteristics of probiotics. One of the most important characteristics of probiotic bacteria is bile

tolerance since it affects the bacteria's capability to serve as a probiotic by determining whether they can survive in the small intestine. Although it seems that strains differ in their ability to tolerate bile, Lactobacilli may eventually become tolerant to the presence of bile salts. Resistant derivatives can be created by subculturing vulnerable wild type bacteria in bile at progressively greater concentrations (Ruiz, Margolles and Sánchez, 2013). The analysis of bile salt resistance in Lactobacillus isolated from ice cream involves understanding the genetic and functional mechanisms that contribute to tolerance. Studies on Lactobacillus strains have identified specific genes associated with bile salt tolerance, such as those involved in the phosphotransferase system, carbohydrate metabolism, and the ATP-binding cassette transporter. Research has shown that Lactobacillus strains exhibit varying levels of tolerance to bile salts, with some strains displaying enhanced resistance mechanisms (Chun-Chiang et.al., 2022; Qiqi et.al., 2021). Additionally, the presence of bile salt hydrolase (BSH) activity in probiotic strains like Lactobacillus plantarum and Lactobacillus gasseri has been linked to their ability to lower serum cholesterol levels and survive in the gastrointestinal tract (Jimmy et.al, 2021; Hiroyuki et.al., 2021). Furthermore, proteomic analysis has revealed that whey powder enriched with milk fat globule membrane proteins can enhance the survival of Lactobacillus plantarum under bile stress conditions by promoting various cellular processes and repair mechanisms (Gangyi et. al, 2024). These findings collectively contribute to a deeper understanding of the bile salt resistance mechanisms in Lactobacillus strains isolated from ice cream.

Bile facilitates in the digestion and absorption of lipids, and through its antibacterial effects, it may also damage the cell membrane's phospholipids and disturb the homeostasis of the cell. It is generally accepted that BSHs contribute greatly to the bacteria that encodes their ability to tolerate bile salts and that there are considerable variances across species' tolerance levels to bile salt treatment, indicating that Lactobacillus may have a variety of resistance mechanisms (Chen et al., 2022). Probiotics' tolerance to bile has been shown to depend on the type of bile and the species, and the average bile concentration is thought to be 0.3% (w/v).

Bile salt resistance plays a crucial role in determining the survival of Lactobacillus species in ice cream. Research has shown that probiotic cultures in ice cream, such as Lactobacillus acidophilus, L. agilis, and L. rhamnosus, exhibit high resistance to bile salts, acidic conditions, and antibiotics, ensuring their viability (Gülden, Hakanand Aynur, 2006). Bernard, Joshi and Andl (2023) study also shows that Lactobacillus species, such as L. plantarum and L. acidophilus, exhibit bile resistance and adaptation to sub-lethal concentrations of bile salts, aiding in their survival in bile-rich environments like the gastrointestinal tract. Encapsulation techniques, like extrusion and emulsion, have been found to effectively protect encapsulated probiotic cells from freezing injury during ice cream processing, enhancing their survival in low pH environments (Andhini, Endang and Tyas, 2012). These findings collectively underscore the significance of bile salt resistance in maintaining the viability of Lactobacillus species in ice cream, ensuring their beneficial effects upon consumption.

In the current investigation, it was discovered that the majority of the chosen strains were resistant to 0.1% (w/v) bile salts, whereas the viable bacterial counts decreased with higher bile salt concentrations. However, several prior investigations also noted that many Lactobacilli were resistant to concentrations of bile salts between 0.3-0.5%, which aided the probiotic's easy colonisation of the host intestine (Feng et

al., 2017). All 5 samples can survive up to a great extent since high absorbance values were detected from spectrophotometry. Figure 6 depicts the bile salt resistance of Lactobacillus in the 5 samples at 0.1%, 0.2% and 0.3% bile salt concentrations along with the variation (standard deviation) respectively. The significance of different bile concentrations lies in their ability to simulate the varying conditions that probiotics may encounter within the gastrointestinal tract. Lower concentrations, such as 0.1%, resemble the initial exposure in the upper digestive system, while higher concentrations, like 0.3%, mimic the more challenging environments found deeper in the intestines. These varying levels help determine the robustness and adaptability of probiotic strains, ensuring their survival and efficacy in promoting gut health (Ayyash et al., 2021). Sample A and D showed high bile salt resistance to 0.3% bile concentration while sample B showed resistance to 0.1%. Prasad et al. (1998), chose four resistant strains (three of dairy and one of human origin) in screening research on 200 lactic acid bacterial strains and assessed bile resistance in a bile concentration range of 0 to 1% with an increase of 0.2% each time. The species was shown to be able to live in the presence of quite high bile salt concentrations like the current study confirming the observation. Sample C and E had the highest resistance to 0.2% which aligns with previous study (Niazi Amraii et al., 2014) where seven acid-resistant species were tested for their capacity to develop in the presence of 0.2% bile salts. All the stains were extremely resistant to bile salts, with a maximum resistance of 152.93%. Overall, bacteria can survive in all concentrations of bile salt and has relatively good resistance to 0.3% bile salt concentration which is the ideal bile salt concentration found in the human body. Results obtained in this study were consistent with previous research findings (Hassanzadazar et al., 2012; Başyiğit, Kuleaşan and Karahan, 2006).

Figure 7 depicts the ANOVA table obtained through carrying out a One-way ANOVA for statistical analysis which calculated a p value of 0.565. The obtained p-value is greater than 0.05, concluding that the data obtained is not statistically significant. This indicates that, while fluctuating resistance was observed, it was not substantial enough to conclude that there is a variation in bile tolerance at different bile salt concentrations.

CONCLUSION

The bile salt resistance capacity tested in the study proves that lactobacillus in all five samples of ice cream have good resistance against bile salts, though there is no significant variation in bile tolerance at different bile salt concentrations. The findings emphasise importance of incorporating Lactobacillus with bile tolerance in food to deliver desired benefits to the consumers.

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